Age Dependence of Pheromone Production by the Boll Weevil (Coleoptera: Curculionidae)

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ABSTRACT Factors influencing pheromone production by the boll weevil, *Anthonomus grandis* Boheman, have been extensively studied, yet recent research using new methods suggests much remains unknown in this regard. The studies reported herein examined age-related changes in production and composition of pheromone at ages from 0 to 6 d, and at 3, 6, 9, and 12 d, respectively, and evaluated the association between accessory gland condition and pheromone production. Estimates of pheromone from extracted feces were near the upper values previously reported. However, ≈95% of the total pheromone was obtained from headspace collections. Based on feces extractions, pheromone production increased with age until the sixth day, while headspace collections indicated an increase in production until the ninth day of adulthood. The boll weevil pheromone is composed of two alcohols (components I and II) and two aldehydes (components III and IV), and ratios of these components changed with age. Component I was dominant for the first days of adult life, but the composition subsequently stabilized at ≈42.5:42.5:5:10 (I:II:III:IV). Also, high levels of pheromone production were associated with well-developed accessory glands, while weevils with small, transparent glands produced little or no pheromone. These results demonstrate production of pheromone in greater quantities, and at earlier ages, than was previously recognized. Additional study using the methods reported herein should provide improved understanding of the dynamics of boll weevil pheromone production that will facilitate ecological interpretation of field data and improvements in trapping systems.

KEY WORDS *Anthonomus grandis*, boll weevil, pheromone, component ratio, volatile collection

THE PHEROMONE TRAP HAS become a tool of critical importance to the management of the boll weevil, *Anthonomus grandis* Boheman, in cotton, *Gossypium hirsutum* L. Eradication programs rely almost exclusively on this trap for detecting boll weevil infestations, evaluating program progress, and indicating the need for insecticide treatments. Despite extensive research efforts to understand the chemical ecology of the boll weevil (Hardee 1972, Hardee and Mitchell 1997), a recent report of preliminary research (Spurgeon and Marshall 2000) suggests that much remains to be learned regarding the dynamics of boll weevil pheromone production.

The boll weevil pheromone consists of two terpene alcohols (components I and II; (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol and *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol, respectively) and two terpene aldehydes (components III and IV; *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde and *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde, respectively) (Tumlinson et al. 1969). Early estimates of boll weevil pheromone production were largely qualitative and relied on responses of weevils to fed males in laboratory assays or traps in the field (Hardee 1970, Klassen and Earle 1970, Earle and Leopold 1975). Subsequently, quantitative estimates of pheromone production were obtained by gas chromatography of fecal extracts (Hedin et al. 1974, McGovern et al. 1976, McKibben et al. 1976, Dickens et al. 1988). However, use of these methods was based on the assumption that pheromone produced by the boll weevil was predominantly, if not entirely, contained in the feces. Because the amount of feces produced daily by a single boll weevil was small, weevils were typically held in large groups for periods of one to several days for collection of feces. Consequently, these methods could not provide an estimate of the variability in pheromone production among individual weevils, and did not account for pheromone volatilized from the feces during the collection period.

Chang et al. (1988, 1989) circumvented the assumption that most pheromone was contained in the feces by estimating the pheromone content of headspace samples. However, their headspace samples were obtained from groups of 60 or 100 laboratory-reared boll weevils. Thus, their methods also provided no estimate of variability in pheromone production among weevils.

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Spurgeon and Marshall (2000) conducted preliminary experiments to examine the pheromone production of individual weevils. These authors obtained estimates of pheromone from the feces that were consistent with earlier reports, but also reported that >94% of the total pheromone collected was obtained from the headspace. Thus, levels of pheromone production indicated in previous reports likely represent severe underestimates of actual production. The objectives of the present studies were to reexamine the preliminary results of Spurgeon and Marshall (2000) by reproducing their evaluation of boll weevil pheromone production during the first 6 d of adulthood, and to extend those findings by investigating the temporal changes in pheromone production with increasing weevil age through 12 d.

Materials and Methods

Experimental Weevils. Adult weevils of known age were reared from infested squares (flower buds) collected from cotton plants. Groups of 200–300 infested squares were placed in 20 by 20 by 20-cm screened Plexiglas cages and held at 29.4 ± 1°C under a 13:11 [L:D] h photoperiod in an environmental chamber. Beginning 5 d after square collection, squares were examined daily and pupae were removed. Pupae were held in groups of 35–50 on a layer of moistened vermiculite in 100 by 15-mm petri plates and under the same conditions as the infested squares. Pupal plates were checked twice daily for newly eclosed adults which were removed and sexed using the method of Sappington and Spurgeon (2000). Only males that were sufficiently sclerotized to walk before 1000 hours (CDT) were used in the pheromone production assays.

Pheromone Collection. Neckless wide-mouth bottles with Teflon-lined lids (120-ml, Qorpak, Bridgeville, PA) were used to contain the weevils during pheromone collection. Two 1.15-cm diameter holes were drilled in the lid of each vessel. Each hole was fitted with 0.95-cm to 0.64-cm Teflon reducing union (Swagelock, Solon, OH), with the large end of the union inside the lid to provide an air-tight seal.

A trap column and a volatile collection column were attached to each collection vessel through the reducing unions. These 20.3 by 0.64-cm (length × outside diameter) glass columns (Envirochem, Lancaster, PA) were each packed with a 5-cm bed of Super Q resin (Alltech Associates, Deerfield, IL) held in place by glass wool. The trap column removed volatiles from air entering the collection vessel while the volatile collection column collected pheromone from air exiting the vessel. The collection columns of eight vessels were connected to a diaphragm vacuum pump through a manifold. Vacuum was adjusted to ≈68 kPa. Air flow through the vessels was regulated at ≈1 liter/min by individual flow meters between the collection vessels and the manifold. The collection vessels were housed in a fume hood maintained at 27 ± 2°C by a thermostatically controlled oscillating heater. Light was provided by two 40-W fluorescent bulbs controlled by an electric timer to provide a photoperiod of 13:11 [L:D] h.

At the end of each 24-h collection period, pheromone obtained from the headspace was eluted from each collection column into a gas chromatography (GC) vial with enough methylene chloride (GC grade) to result in a 1.0-ml eluant volume. Contents of each vial were mixed by agitation and duplicate 1-μl injections were analyzed by GC. Pheromone from the feces was obtained by brushing the feces from the food source (cotton square) into the collection vessel where it was extracted by gentle swirling with 1.0 ml of GC-grade hexane. Pheromone content of the feces was also estimated by GC from duplicate 1-μl injections of the extract. Pheromone content from headspace collections and feces extractions were each calculated from the averages of their respective duplicate injections. Spurgeon and Marshall (2000) previously determined that collection efficiency of these techniques was ≈95%, so estimates of the quantities of pheromone collected were not corrected to account for recovery efficiency.

Samples were analyzed using a Hewlett–Packard 5890 series II GC (Hewlett–Packard, Palo Alto, CA) equipped with a DB-5 column (30 m × 0.25 mm, length × i.d.; J&W Scientific, Folsum, CA) and a flame-ionization detector. Injector temperature was 200°C, detector temperature was 300°C, and flow rate was 2.0 ml/min. An initial column temperature of 60°C was maintained for 7 min, then increased to 110°C at 30°C/min, decreased to 100°C at 2°C/min and maintained at that temperature for 5 min, and finally increased to 300°C at 10°C/min and held at that temperature for 10 min. Total GC run time was 48.67 min. Concentrations of each pheromone component were calculated by comparing respective areas under the sample peaks to corresponding areas for external standards of a known concentration of Grandlure (ISP Fine Chemicals, Columbus, OH). The total amount of pheromone eluted from a collection column or extracted from feces samples was calculated by adding the quantities of the four individual components.

Accessory Gland Ratings. At the end of the last 24-h pheromone collection period for each weevil, accessory gland condition was assessed by dissection under a stereomicroscope at 15–20 ×. Accessory glands were assigned to one of four classes derived from previous observations of their variability in color and size (Spurgeon 2001). Class 1 glands are very small and transparent, and are not apparent to the casual observer especially when well-developed fat bodies are present. Class 2 glands are also transparent, but are larger and more readily apparent than class 1 glands. Class 3 glands are slightly to considerably larger than class 2 glands, and contain a gray, gelatinous material in the lumen of part of their length. Class 4 glands are large enough to visually dominate the volume of the abdomen, and contain the gray material throughout the length of the lumen.

Pheromone Production between 0 and 6 d of Age. In each repetition of the experiment, eight newly eclosed male weevils were transferred to individual

volatile collection vessels. Each vessel contained a fresh, uninfested square with bracts intact and with a bud diameter between 6 and 7 mm. Deionized water was supplied in a 7.5-ml plastic vial closed with a cotton wick. Squares were replaced each day when pheromone was collected from the adsorbent columns and feces. Each daily pheromone collection period was as near as possible to 24 h. Collections were terminated after the sixth 24-h period, when weevils were dissected to determine condition of the accessory glands. The experiment was repeated four times between 12 July and 17 August 2000.

Pheromone Production between 3 and 12 d of Age. In each repetition of the experiment, eight newly eclosed weevils were transferred to individual 100 by 15-mm petri plates. Each weevil was furnished a fresh, intact square of 6–7 mm diameter and a short (≈1 cm) section of dental wick saturated with deionized water. Weevils were maintained in an environmental chamber at 29.4 ± 1°C with a photoperiod of 13:11 [L:D] h. Squares were replaced daily before 1000 hours (CDT).

At the beginning of the 3rd, 6th, 9th, and 12th 24-h periods after adult eclosion, the weevils were transferred to individual volatile collection vessels, each containing a fresh square and a water source. After 24 h, pheromone was collected as previously described and the weevils were returned to their respective petri plates until the next pheromone collection period. Immediately after the final collection period (day 12), weevils were dissected to determine accessory gland condition. The experiment was repeated four times between 21 August and 22 September, 2000. Repetitions of the experiment overlapped because any given cohort of weevils required use of the collection vessels only on every third day.

Statistical Analysis. The data from both experiments were examined in similar but separate analyses. The influences of pheromone source (headspace, feces), repetition of the experiment (run), and weevil age on pheromone production were examined by repeated measures analysis of variance (ANOVA) using the REPEATED statement of the procedure PROC GLM (SAS Institute 1998). Day of weevil age was the repeated factor, and the respective models also included terms for all possible interactions of main effects. When the test for sphericity rejected the Huynh-Feldt condition at $P < 0.0001$, the statistical significance of the repeated factor and any interactions containing that factor was assessed using the Wilk's Lambda statistic. When the Huynh-Feldt condition was rejected at $0.05 > P > 0.0001$, probability levels adjusted by the Greenhouse-Geisser epsilon were used. Because some weevils did not survive the entire pheromone collection period, sample sizes of the respective runs of the experiments were unequal. Thus, differences in pheromone production among runs of the experiment within respective weevil ages were examined using the TUKEY option of the MEANS statement of PROC GLM (SAS Institute 1998). Differences in pheromone collected between sources (headspace, feces) within weevil ages were examined using the REGWQ option.

Changes in pheromone component ratios with increasing weevil age were examined by multivariate analyses using the MANOVA statement of PROC GLM (SAS Institute 1998). Component ratio data for each study and source (headspace, feces) of pheromone were analyzed separately. Dependent variables were the respective proportions of the total pheromone represented by each of the four components. Proportions were arcsine-square root transformed (Zar 1984) before analysis. The models contained terms for weevil age, repetition of the experiment (run), and the interaction of these effects. Because not all weevils produced pheromone on a given day, comparisons of the proportions of a given component among levels of each main effect were examined using the TUKEY option of the MEANS statement of PROC GLM (SAS Institute 1998).

Relationships between pheromone production and accessory gland condition were examined by ANOVA. Separate analyses were conducted for pheromone recovered from the headspace and feces, respectively. Because accessory gland condition can change over a 24-h period (unpublished data), the amount of pheromone recovered during the final 24-h collection period was the dependent variable. The respective models contained terms for run of the experiment, accessory gland condition, and their interaction. Differences in pheromone production between levels of run or accessory gland condition were examined using the TUKEY option of the MEANS statement (SAS Institute 1998). When the run by accessory gland condition interaction was significant, differences among combinations of these factors were examined using the PDIFF and ADJUST = TUKEY options of the LSMEANS statement (SAS Institute 1998).

Results

Pheromone Production between 0 and 6 d of Age. On average, considerably more pheromone was obtained from headspace collections (26.88 μg weevil^{-1} day^{-1}) than from feces extractions (1.16 μg weevil^{-1} day^{-1}) ($F = 40.28$; df = 1, 54; $P < 0.01$), and this relationship was consistent among runs of the experiment (run by source interaction; $F = 1.25$; df = 3, 54; $P = 0.30$). Levels of pheromone production were also generally similar among runs of the experiment ($F = 1.51$; df = 3, 54; $P = 0.22$). Overall, the proportion of total pheromone that was obtained from the headspace collections ranged from 95.1% (run 4) to 96.2% (run 2).

Pheromone production also increased with weevil age (Wilk's Lambda = 0.355; $F = 18.15$; df = 5, 50; $P < 0.01$), but the extent of this increase varied with pheromone source (age by source interaction; Wilk's Lambda = 0.368; $F = 17.16$; df = 5, 50; $P < 0.01$). Pheromone was recovered from headspace samples at earlier ages, and increased to much higher levels, compared with pheromone from feces extractions (Fig. 1). The day by run interaction (Wilk's Lambda = 0.513; $F = 2.53$; df = 15, 138.43; $P < 0.01$) indicated that patterns of daily pheromone production varied among

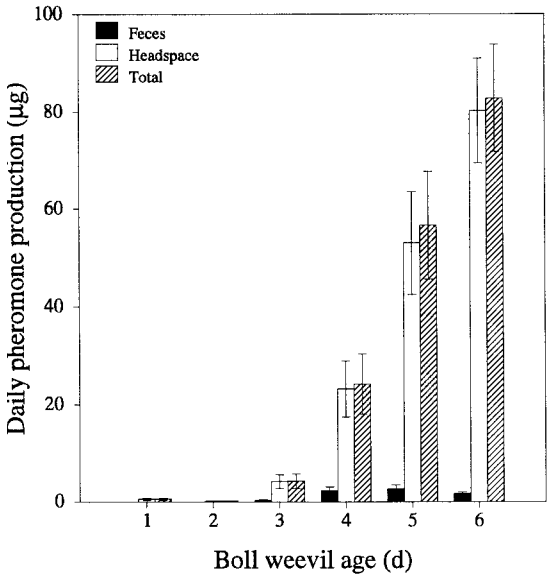

Fig. 1. Daily pheromone production (mean ± SEM) by male boll weevils from adult eclosion to 6 d of age indicated by feces extractions and headspace collections.

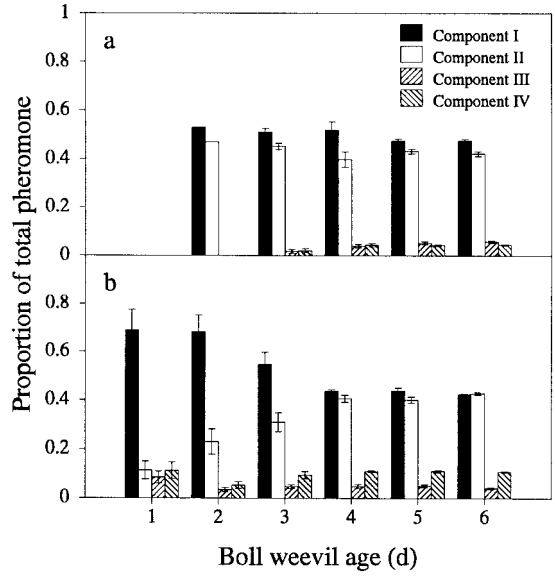

Fig. 2. Age-dependent changes in boll weevil pheromone composition (mean ± SEM) from adult eclosion to 6 d of age; (a) feces extractions, (b) headspace collections. Component I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; component II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; component III, *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and component IV, *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde.

repetitions of the experiment. In particular, pheromone production was initiated earlier in the first two runs than in the final two runs of the experiment. Over all repetitions, ≈32% of weevils produced detectable quantities of pheromone during the first 24-h period after adult eclosion, and >90% of weevils were producing pheromone by the sixth day of age.

The composition of pheromone obtained from extractions of feces was dominated by components I and II, but varied with run of the experiment (Wilk's Lambda = 0.710; $F = 1.90$; df = 12, 164.33; $P = 0.04$) and weevil age (Wilk's Lambda = 0.406; $F = 4.08$; df = 16, 190.05; $P < 0.01$). Further, the variations in component ratios among weevil ages were not consistent among runs (Wilk's Lambda = 0.388; $F = 1.87$; df = 36, 234.08; $P < 0.01$). Univariate tests of individual components indicated that the changes with increasing weevil age in the proportion of total pheromone represented by each component varied among runs only in the case of component III (day by run interaction; $F = 2.71$; df = 9, 65; $P < 0.01$).

Pheromone was not detected in the feces during the first 24-h period after adult eclosion, and only components I and II were present during the second 24-h period. The proportion of total pheromone represented by component I tended to decrease with increasing weevil age ($F = 2.98$; df = 4, 65; $P = 0.03$), but these differences were too small for detection by the means separation procedure (Fig. 2a). Similar changes in the proportions of component II were not detected ($F = 2.00$; df = 4, 65; $P = 0.10$), but the proportions of components III ($F = 10.06$; df = 4, 65; $P < 0.01$) and IV ($F = 5.05$; df = 4, 65; $P < 0.01$) both increased with weevil age (Fig. 2a).

The composition of pheromone obtained from headspace collections was also dominated by components I and II, and varied little among runs of the experiment (Wilk's Lambda = 0.870; $F = 1.05$; df = 12, 233.12; $P = 0.41$). Composition did, however, vary among weevil ages (Wilk's Lambda = 0.403; $F = 4.61$; df = 20, 292.81; $P < 0.01$) (Fig. 2b). Further, the analyses did not indicate that the age-related changes in pheromone composition differed among runs of the experiment (age by run interaction; Wilk's Lambda = 0.512; $F = 1.21$; df = 52, 342.93; $P = 0.17$).

The most marked age-related changes in the composition of pheromone from the headspace samples were the decreasing proportions of component I during the first 4 d ($F = 11.01$; df = 5, 91; $P < 0.01$) and a corresponding increase in component II during the same period ($F = 15.74$; df = 5, 91; $P < 0.01$) (Fig. 2b). The proportion of pheromone represented by component III varied little with weevil age ($F = 1.26$; df = 5, 91; $P = 0.29$), and was generally between 4 and 5%. Although the proportion of component IV was lower on day 2 (5.3%) than on other days (9.5–11.3%) ($F = 5.60$; df = 5, 91; $P < 0.01$), no general trend in the proportion of this component was observed with respect to weevil age.

Although there was a numerical trend for increasing pheromone content of the feces of 6-d-old weevils with increasing accessory gland development, the overall ANOVA did not indicate a statistical relationship ($F = 1.55$; df = 8, 22; $P = 0.20$; Table 1). However, levels of pheromone production indicated by headspace collections during the sixth day of adulthood differed among accessory gland classes ($F = 12.76$;

Table 1. Influence of accessory gland condition on daily pheromone production (mean ± SEM) of 6-d-old male boll weevils indicated by headspace collections and feces extractions

Accessory gland class[a]	*n*	Pheromone in feces (μg)	Pheromone in headspace (μg)
1	2	0.00 ± 0.00	0.00 ± 0.00c
2	3	0.23 ± 0.12	1.81 ± 1.22c
3	21	1.65 ± 0.33	84.81 ± 9.00b
4	5	3.44 ± 1.07	140.30 ± 35.29a

Means followed by different letters are significantly different at $\alpha = 0.05$, Tukey Studentized Range Test.

[a] Accessory gland classes were: 1, very small and transparent, not apparent; 2, transparent, but larger and more apparent than class 1; 3, larger than class 2 and containing a gray material in the gland lumen; 4, large enough to visually dominate the volume of the abdomen and containing the gray material throughout the gland lumen.

df = 3, 22; $P < 0.01$; Table 1). In addition, the run by accessory gland class interaction was significant ($F = 4.64$; df = 2, 22; $P = 0.02$), indicating some variability among runs of the experiment in the relationship between gland class and pheromone production. In general, class 3 and class 4 accessory glands were associated with high levels of pheromone production while classes one and two were associated with little or no production of pheromone.

Pheromone Production between 3 and 12 d of Age. As in the case of the previous study, average daily estimates of pheromone production were greater for headspace samples (37.50 μg/weevil) than for fecal extractions (1.73 μg/weevil) ($F = 84.97$; df = 1, 46; $P < 0.01$), and these differences were consistent among runs of the experiment (run by source interaction; $F = 0.23$; df = 3, 46; $P = 0.90$). The proportions of total pheromone that were contributed by the headspace collections were similar to those in the previous experiment, ranging from 93.8% (run 4) to 97.7% (run 3).

Analyses of daily pheromone production indicated a significant effect of weevil age ($F = 29.39$; df = 3, 138; *G-G Adjusted* $P < 0.01$) that was consistent among experimental runs (age by run interaction; $F = 1.50$; df = 9, 138; *G-G Adjusted* $P = 0.17$) but varied with source of the pheromone (age by source interaction; $F = 26.03$; df = 3, 138; *G-G Adjusted* $P < 0.01$). In general, pheromone production indicated by headspace collections increased with weevil age until the ninth day, while pheromone content of the feces peaked on day 6 and diminished thereafter (Fig. 3). Thus, under the conditions of the experiment, peak pheromone production occurred around the ninth day of adulthood. Over all runs of the experiment, ≈56% of weevils were producing pheromone by day 3, 97% by day 6, and 100% by days 9 and 12.

The pheromone obtained from frass was dominated by components I and II. Component ratios varied among runs of the experiment (Wilk's Lambda = 0.689; $F = 2.34$; df = 12, 185.49; $P < 0.01$), and among weevil ages (Wilk's Lambda = 0.460; $F = 5.27$; df = 12, 185.49; $P < 0.01$). In this experiment, proportions of both component II ($F = 2.80$; df = 3, 73; $P < 0.05$) and component III ($F = 5.55$; df = 3, 73; $P < 0.01$) varied among runs of the experiment. But these differences

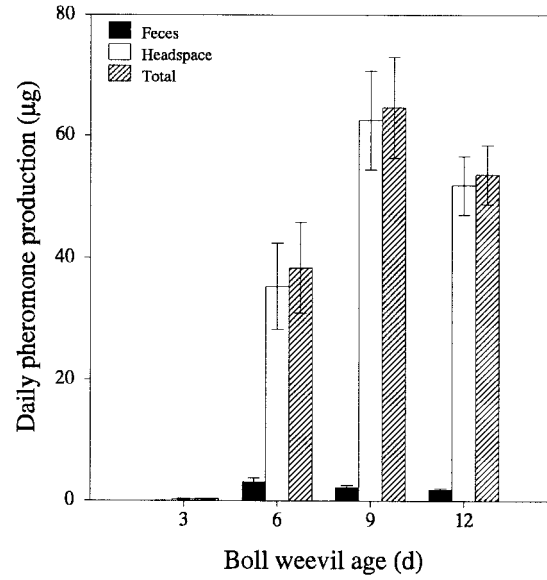

Fig. 3. Daily pheromone production (mean ± SEM) by male boll weevils at ages of 3, 6, 9, and 12 d indicated by feces extractions and headspace collections.

were not sufficient to produce a significant age by run interaction (Wilk's Lambda = 0.643; $F = 1.38$; df = 24, 245.41; $P = 0.12$).

Only components I and II were detected in feces produced during the third day of adulthood. The proportion of component I decreased between days 3 and 6 ($F = 20.84$; df = 3, 73; $P < 0.01$) while the proportion of component II increased during this same period ($F = 12.57$; df = 3, 73; $P < 0.01$) (Fig. 4a). The significant influence of weevil age on the proportions of components III ($F = 7.05$; df = 3, 73; $P < 0.01$) and IV ($F = 7.77$; df = 3, 73; $P < 0.01$) appeared to be caused primarily by their absence from the samples on day 3. No further changes in the proportions of any components were observed after day 6.

The analysis did not detect differences among runs of the experiment in the component ratios of headspace samples (Wilk's Lambda = 0.809; $F = 1.73$; df = 12, 248.99; $P = 0.06$). Component ratios varied among weevil ages (Wilk's Lambda = 0.763; $F = 2.23$; df = 12, 248.99; $P = 0.01$), but the changes with increasing age were not consistent among runs (Wilk's Lambda = 0.561; $F = 1.85$; df = 32, 348.25; $P < 0.01$). The proportions of component I decreased between day 3 and day 9 ($F = 3.75$; df = 3, 97; $P = 0.01$) (Fig. 4b), and these changes were consistent among runs ($F = 1.16$; df = 8, 97; $P = 0.33$). During this same period, the proportions of component II increased ($F = 6.43$; df = 3, 97; $P < 0.01$). Overall, the proportions of component III decreased between day 3 and day 6 ($F = 2.93$; df = 3, 97; $P = 0.04$), but these changes were not consistent among runs (age by run interaction; $F = 2.17$; df = 8, 97; $P = 0.04$). Examination of individual runs indicated in the third run of the experiment the proportion of component III increased between day 3 and 6, only to

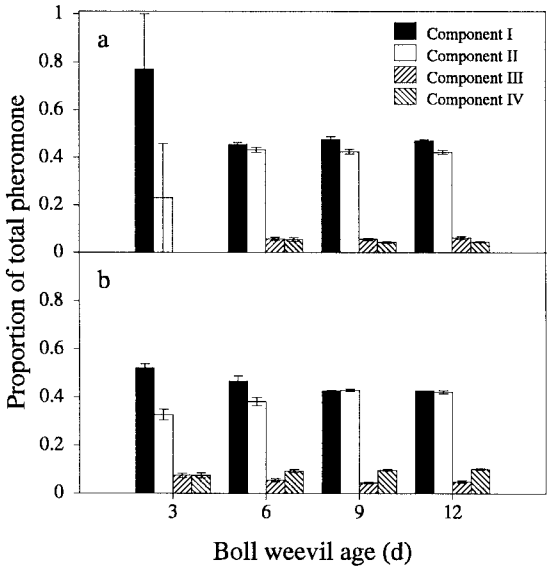

Fig. 4. Age-dependent changes in boll weevil pheromone composition (mean ± SEM) at ages of 3, 6, 9, and 12 d; (a) feces extractions, (b) headspace collections. Component I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; component II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; component III, *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and component IV, *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde.

decrease again by day 9. In other runs, the proportions of component III decreased between days 3 and 6 (runs two and 4), or remained relatively unchanged with increasing weevil age (run 1). Proportions of component IV did not change significantly with increasing weevil age ($F = 0.95$; df = 3, 97; $P = 0.42$) (Fig. 4b).

All weevils surviving the experimental period had produced pheromone by the twelfth day, and only accessory gland classes three ($n = 11$) and four ($n = 15$) were observed in dissections. Numerically, the average quantity of pheromone obtained from the headspace collections was slightly higher for gland class 4 (mean ± SEM, 56.65 ± 4.93 μg) than for gland class 3 (mean ± SEM, 49.68 ± 8.50 μg). In contrast, the mean quantity of pheromone extracted from feces was higher for gland class 3 (mean ± SEM, 2.07 ± 0.52 μg) than for gland class 4 (mean ± SEM, 1.64 ± 0.24 μg). However, analyses did not indicate that these differences were statistically significant (feces, $F = 2.45$; df = 7, 18; $P = 0.06$; headspace, $F = 1.45$; df = 7, 18; $P = 0.25$).

Discussion

Estimates of daily pheromone production indicated by fecal extractions were toward the upper limits of those previously reported. Hedin (1976) reported the average daily production of pheromone in the feces was ≈1.3 μg. Gueldner and Wiygul (1978) estimated daily pheromone production was ≈1 μg/weevil on the twelfth day of adulthood. Villavaso et al. (1983) reported an average daily production of 0.443 μg over a 7-d period, while Dickens et al. (1988) indicated that daily pheromone production reached a level of ≈0.5 μg/weevil by the fifth day of adulthood. The somewhat higher levels of pheromone production indicated in the fecal samples of the current study may have resulted from differences in the diet supplied compared with previous studies. Hedin (1976) does not indicate the diet used while Villavaso et al. (1983) fed plugs of artificial diet. Gueldner and Wiygul (1978) and Dickens et al. (1988) fed cotton squares, but at a rate of one square per five weevils daily. This diet is one that has been used to induce diapause in recent studies (Spurgeon and Raulston 1997, 1998a, Spurgeon and Esquivel 2000), and thus may not have supported an optimal level of pheromone production. In contrast, Spurgeon and Marshall (2000) and the current study used a feeding regime known to result in a high degree of reproductive commitment (Spurgeon and Raulston 1998b). Given the observed relationship between pheromone production and accessory gland development, which is closely tied to reproductive development (unpublished data), it seems likely that differences in feeding regimes could account for the different estimates of pheromone production. Further, the differences between the present findings and those of Spurgeon and Marshall (2000) may have been caused by the greater control over square size that was exercised in the current study.

Regardless of between-study comparisons of the pheromone content of boll weevil feces, the vast majority of pheromone recovered in the present studies was obtained from the headspace. Estimates of pheromone indicated by the headspace collections were also much higher than those previously reported for similar techniques. Chang et al. (1988, 1989) estimated daily levels of pheromone production of 1.3 μg and 4.2 μg/weevil, respectively. However, these levels of daily production were extrapolated from the results of 8-h headspace sampling periods. Further, their techniques measured pheromone produced by groups of 60–100 laboratory-reared weevils that were supplied a comparatively limited diet. Thus, differences in the strains of weevils examined, the potential for an effect of crowding, and the aforementioned influence of diet are all factors that could possibly account for the lower rates of pheromone production estimated by Chang et al. (1988, 1989).

It has been widely accepted that the boll weevil releases pheromone from the feces. Gueldner and Wiygul (1978) suggested the feces serve the role of a controlled release substrate, and speculated as to the ecological implications of this function. In light of the present results which indicate that the feces contain only a very small proportion of the total pheromone produced, it seems unlikely that feces serve such a role, and more likely that they are simply contaminated during the release of pheromone. Additional studies to more fully investigate this aspect of boll weevil pheromone production would seem warranted.

The changes in pheromone production with increasing weevil age that were indicated by headspace collections were different from those indicated by fecal extractions. McKibben et al. (1976) indicated that pheromone production during the first 6 d of adulthood were too low for quantitation, and Hedin (1976) stated that pheromone production was very limited for the first 5 d after adult emergence. Gueldner and Wiygul (1978) reported that pheromone production generally increased each day through the eighteenth day of adult age, and suggested that peak pheromone production followed a 6-d cycle. Although results of the current study indicated that, with the exception of a single weevil, pheromone was not detected in the feces until the third day of adulthood, headspace samples indicated a sizeable proportion of weevils produced measurable quantities of pheromone during the first 24-h period after adult eclosion. Also, total pheromone production generally increased through the ninth day of adult age, and comparatively large amounts of pheromone were produced as early as 3 d after adult eclosion. Finally, results of the current study do not support occurrence of the long-term, 6-d cyclic pattern in pheromone production suggested by Gueldner and Wiygul (1978), and suggest that additional studies using the techniques described herein may provide a more accurate and meaningful understanding of the influence of weevil age on daily patterns of pheromone production.

Estimates of component ratios of the boll weevil pheromone obtained from fecal extractions have varied widely. Tumlinson et al. (1970) reported approximate component ratios of 52.4:39.3:4.1:4.1 (I:II:III:IV). Hedin et al. (1974) suggested that young males were not capable of synthesizing the aldehyde components of the pheromone because components III and IV were not found in males during the first 6 d after eclosion, and reported the component ratio over the first 31 d of adult life was 6:6:2:1. McGovern et al. (1976) combined estimates of components III and IV, and reported component ratios of 46:10:44 for overwintered weevils, and 46:22:32 for first generation field weevils. Irrespective of these estimates, the component ratio evaluated in pheromone lures by Leggett et al. (1989) (30:40:15:15; I:II:III:IV) is currently used in commercial lures. In contrast, results of the current study indicate that male weevils are capable of synthesizing all components of the pheromone by the earliest ages at which pheromone production can be detected, although component I dominates the pheromone composition for the first few days of adult life. In addition, pheromone composition changes markedly during the first days of adult life, but then stabilizes at a ratio of ≈47:43:6:4 (feces extractions) and 42.5:42.5:5:10 (headspace collections). Because of the overwhelming contribution of the headspace collections to the total pheromone recovered, the component ratio of total pheromone collected was indistinguishable from that of the headspace collections. It should be noted that pheromone component ratios varied somewhat among runs of both experiments, and may be influenced by diet and other factors besides adult age. Additional studies will be required to fully investigate the factors influencing both the quantities and quality of pheromone produced by the male boll weevil.

Hedin (1977) suggested the weevil gut played a major role in pheromone synthesis or release. However, accessory glands were not examined in his study, and the observations reported here of an association between accessory gland condition and pheromone production suggests a role of these glands in either pheromone production, processing, or release. Additional biochemical studies will be necessary to elucidate what role, if any, the accessory glands play in the dynamics of boll weevil pheromone production.

Irrespective of whether the accessory glands participate in the production or release of pheromone, or are simply a correlate of pheromone production capability, the combined results of the two present experiments suggest there is no justification for maintaining the current accessory gland rating system. Rather, it should be sufficient to classify glands as either not associated with pheromone production (combined classes 1 and 2) or as associated with pheromone production (combined classes 3 and 4). This suggestion also greatly reduces the subjective nature of the rating system because gland classes 1 and 2 are easily distinguished from classes 3 and 4.

Although the analyses in the current report suffered from low sample sizes with regards to gland classes 1 and 2, additional studies have confirmed this relationship even in the presence of hypertrophied fat bodies (Young and Spurgeon 2002). Young and Spurgeon (2002) combined accessory gland classes 1 and 2, and classes 3 and 4, and designated the two resulting classes as "undeveloped" and "developed," respectively. In two separate experiments they estimated mean daily pheromone production by weevils with "undeveloped" and "developed" accessory glands as <0.5 μg/weevil ($n = 2$) and 89.1 μg/weevil ($n = 29$), respectively, at 7 d of age, and 1.6 μg/weevil ($n = 7$) and 86.3 μg/weevil ($n = 47$), respectively, at 9 d of age. This relationship may offer a rapid and inexpensive means of gleaning meaningful ecological information from field studies because the pheromone production status of field- or trap-collected weevils can be rapidly determined by dissection.

The results of the research presented herein are generally consistent with the report of Spurgeon and Marshall (2000). Most notable, those results indicate that pheromone production in the boll weevil occurs earlier and at much higher rates than was previously recognized, but also that this production varies considerably among individual weevils or groups of weevils. The sources of this variability may include genetic differences among individual weevils or cohorts of weevils, subtle influences of temperature, or unidentified characteristics of the food supplied. A complete understanding of the dynamics of boll weevil pheromone production will require additional investigation of these and other factors. In that light, the pheromone collection apparatus and procedures used in this study appear uniquely suitable for that purpose, and should

prove invaluable to further study of the ecology of boll weevil pheromone production.

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